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Zoological Research

Phylogenetic Relationship of the Firefly, *Diaphanes pectinealis* (Insecta, Coleoptera, Lampyridae) Based on DNA Sequence and Gene Structure of Luciferase

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Abstract: *Diaphanes* is the fourth largest genus in Lampyridae, but no luciferase gene from this genus has been reported. In this paper, by PCR amplification of the genomic DNA, the luciferase gene of *Diaphanes pectinealis*, which is the first case from *Diaphanes*, was identified and sequenced. The luciferase gene from *D. pectinealis* spans 1958 base pairs (bp) from the start to the stop codon, including seven exons separated by six introns, and encoding a 547-residue-long polypeptide. Its deduced amino acid sequence showed high protein similarity to those of the Lampyrini tribe (93 – 94%) and the Cratomorphini tribe (92%), while low similarity was found with the North American firefly *Photinus pyralis* (83%) of the Photinini tribe within the same subfamily Lampyrinae. The phylogenetic analysis performed with the deduced amino acid sequences of the luciferase gene further confirms that *D. pectinealis*, *Pyrocoelia*, *Lampyris*, *Cratomorphus*, and *Photinus* belong to the same subfamily Lampyrinae, and *Diaphanes* is closely related to *Pyrocoelia*, *Lampyris*, and *Cratomorphus*. Furthemore, the phylogenetic analysis based on the nucleotide sequences of the luciferase gene indicates *Diaphanes* is a sister to *Lampyris*. The phylogenetic analyses are partly consistent with morphological (Branham & Wenzel, 2003) and mitochondrial DNA analyses (Li et al, 2006).

Key words: Firefly; *Diaphanes pectinealis*; Luciferase gene; Gene structure; Phylogeny

栉角雪萤的荧光酶基因结构及其系统发育分析

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摘要: 短角窗萤属是萤科第四大属, 但未见有该属物种荧光酶基因的研究报道。通过对总基因组的 PCR 扩增, 对该属的栉角雪萤荧光酶基因进行了测序分析。基因序列长 1 958 bp。与已知荧光酶基因进行同源性比较后推断, 栉角雪萤的荧光酶基因由 7 个外显子和 6 个内含子组成, 编码 547 个氨基酸残基; 由推导的氨基酸序列进行同源性比较后发现, 栉角雪萤的荧光酶基因与同一亚科中 Lampyrini 族和 Cratomorphini 族分别具有 93—94%和 92%相似性, 而与北美萤火虫 *Photinus pyralis* (Photinini 族)的相似性较低(83%)。系统发育分析进一步表明栉角雪萤与 *Pyrocoelia*、*Lampyris*、*Cratomorphus* 和 *Photinus* 同属于萤亚科, 且与前 3 个属的亲缘关系较近。这在一定程度上与形态(Branham & Wenzel, 2003)及线粒体 DNA(Li et al, 2006)系统发育分析所得结果一致。

关键词: 栉角雪萤; 荧光酶基因; 基因结构; 系统发育
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Fireflies include the beetles of the family Lampyridae (lampyrid fireflies) in a narrow sense, or the beetles of the families Lampyridae and Rhagophthalmidae (rhagophthalmid fireflies) in a broader sense (Li & Liang, 2006). Lampyrid fireflies are well known as luminescent insects, which mainly occur in tropical and subtropical areas, including about 2000 known species in more than 92 genera and subgenera (McDermot, 1966) of eight subfamilies (Crowson, 1972; Lawrence & Newton, 1995). In China, 116 lampyrid species belonging to 13 genera and 5 subfamilies were reported (Li, 2005). Among these Chinese lampyrids, the most enigmatic is a species from Gaoligongshan Mt., Yunnan, Southwest of China, which exhibits the general morphology of *Diaphanes luniger* Motschulsky, i.e. the type of the genus *Diaphanes*, except for its pectinate antennae, which make its phylogenetic position uncertain. Based on our observations on morphology and the references to mitochondrial DNA data (Li et al, 2006), this enigmatic species should be placed in the genus of *Diaphanes* (Lampyrinae: Lampyrini) and described as new to science under the name of *Diaphanes pectinealis* (Li & Liang, paper submitted*).

Firefly photic signals play an important role in sexual communication. Therefore the firefly is a good system for studying sexual selection and speciation (Lloyd, 1971, 1973; Ohba, 1983, 1997). For this reason fireflies have been studied widely with respect to behavior (see Viviani, 2002). More importantly, the luciferase gene, responsible for bioluminescence, has potential applications in molecular biology and medicine as a biomarker (Greer & Szalay, 2002; Viviani, 2002). Recently more attention has been directed towards the study of the luciferase gene, of which 14 lampyrid (three subfamilies, six genera) and one rhagophthalmid genes have been cloned and sequenced so far (Tab. 1). Comparison of the amino acid sequence of the luciferase gene among some species indicated the usefulness of this gene for phylogenetic analysis at the species, or higher taxomic level (Choi et al, 2002). It is regretful, however, that no *Diaphanes* species luciferase gene has been reported.

In this study, in order to investigate the luciferase gene of *Diaphanes* species and the phylogeny of *D. pectinealis* better, we analyzed the gene structure, and compared the sequence with the luciferase cDNA sequence and genes of species from similar genera as listed in Tab. 1.

1 Materials and Methods

1.1 Materials

The firefly, *Diaphanes pectinealis* used in this study was collected at Gaoligongshan Mt., Yunnan, Southwest of China in October, 2003. Extra specimens were pinned or soaked in 75% alcohol as voucher specimens, and deposited in Kunming Institute of Zoology (KIZ), the Chinese Academy of Sciences (CAS), China.

1.2 DNA extraction

Total DNA was extracted according to the phenol-chloroform method (Sambrook et al, 1989). Only the thorax of a single specimen was minced with scissors and digested at 56 ℃ overnight in 600 μL STE (10 mmol/L NaCl, 10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 7.5) plus 60 μL 10% sodium dodecyl sulfate (SDS) and 10 μL 20 mg/mL proteinase K. DNA was purified by saturated phenol extraction once followed by chloroform and isoamyl alcohol (24∶1) extraction twice, and then precipitated with isopropyl alcohol and rinsed with ethanol once. Finally, DNA was dissolved in TE (10 mmol/L Tris-HCl, 1 mmol/L EDTA, pH 8.0).

1.3 PCR amplification and DNA sequencing

The primers used for amplification of the genomic DNA of the luciferase from the *D. pectinealis* were Lam_noc_10 (acg cgc taa tat cat tgc a) and Lam_R (ttc gtt aga ata tag taa acc gaa g), which were designed by authors based on the luciferase cDNA of *Lampyris noctiluca* (Sala-Newby et al, 1996). PCR conditions were as follows: an initial denaturation step at 94 ℃ for 4 min; 35 cycles of 94 ℃ for 20 s, 48 ℃ for 30 s, and 72 ℃ for 3 min; and a final extension step at 72 ℃ for 7 min. PCR products were checked by electrophoresis using 1% agarose gel in 1 × TAE buffer. The PCR products were then purified using DNA Fragment Quick Purification/Recover Kit (Yun Ke Co., China) following the manufacturer's instructions.

DNA sequencing was performed using an automatic sequencer ABI3100. The initially sequencing primers were the same as the PCR amplification primers, with subsequent sequencing being performed by primers designed with reference to previously-deter

*Li XY, Liang XC. A new species of *Diaphanes* Motschulsky (Coleoptera: Lampyridae: Lampyrinae) from Gaoligongshan Mountains of western Yunnan Province, China, with A review of known Chinese species[J]. (submitted)

mined sequences (primer walking).

1.4 Data analysis

With 23 GenBank-registered sequences, initial alignment of the sequences was conducted using Clustal X (Thompson et al, 1997), and confirmation of the alignment was done manually using BioEdit (Version 4.7.8).

Phylogenetic trees were constructed by the Neighbor-Joining (NJ) (Saitoh & Nei, 1987) and Maximum Parsimony (MP) methods (Farris, 1970) using PAUP* 4.0b10 (Swofford, 2002). The accession numbers of the sequences in the GenBank are listed in Tab. 1.

Kimura's two parameters model was selected (Kimura, 1980) and bootstrap of 1000 replicates was performed in the NJ analysis. Maximum Parsimony analysis was performed using heuristic searches with 100 random-addition sequences via Tree-bisection-reconnection (TBR) branch swapping, and support for the resulting nodes was assessed using bootstrap (BP) analysis (1000 replicates) (Felsenstein, 1985). All characters are unordered and weighted equally.

Tab. 1 GenBank accession number of luciferase gene used in this study

Family & subfamily	Tribe	Species	GenBank accession number	References
Ingroups:				
Lampyridae				
Lampyrinae	Photini	*Photinus pyralis*	M15077	De Wet et al, 1985, 1987
	Lampyrini	*Pyrocoelia miyako*	L39928	Ohmiya et al, 1995
		Pyrocoelia rufa	AF328553; AY447202; AY447203	Lee et al, 2001; Li et al, 2003b
		Lampyris noctiluca	X89479; AY447204	Sala-Newby et al, 1996; Li et al, 2003a
		Lampyris turkestanicus	AY742225	Aplipour et al, 2004
		Diaphanes pectinealis	**DQ408300**	**This study**
	Cratomorphini	*Cratomorphus distinctus*	AY633557	Viviani et al, 2004
Luciolinae		*Luciola cruciata*	M26194	Masuda et al, 1989
		Lucioloa lateralis	X66919	Tatsumi et al, 1992; Cho et al, 1999
		Luciola mingrelica	S61961	Devine et al, 1993
		Hotaria parvula	L39929	Ohmiya et al, 1995
		H. unmunsana	AF420006; AF486800	Choi et al, 2002
		H. papariensis	AF486802; AF486803	Choi et al, 2003
		H. tsushimana	AF486801; AF486804	Choi et al, 2003
Photurinae		*Photuris pennsylvanica*	U31240	Li et al, 1997
Outgroups:				
Rhagophthalmidae		*Rhagophthalmus ohbai*	–	Ohmiya et al, 2000
Phengodidae		*Phrixothris viviani*	AF139644	Viviani et al, 1999
		Phrixothris hirtus	AF139645	Viviani et al, 1999

2 Results and Analysis

2.1 The gene structure and sequence

The luciferase gene of *Diaphanes pectinealis* contains 1958 base pairs (bp) from the start to the stop codons. Although the length is different, the luciferase genes in various firefly species usually consist of six introns and seven exons (Li et al, 2003a). Furthermore, the intron boundaries include an invariant GT bases at the intron five boundary and an invariant AG bases at the intron three boundary and are well conserved in the known luciferase genomic sequence of fireflies, such as *Lampyris noctiluca* (Li et al, 2003a), *Pyrocoelia rufa* (Li et al, 2003b), *Hotaria*-group fireflies (Choi et al, 2003) and *Luciola lateralis* (Cho et al, 1999). By comparison with the luciferase cDNA sequences of *Pyrocoelia miyako* (Ohmiya et al, 1995), *P. rufa* (Lee et al, 2001), *Lampyris nociluca* (Sala-Newby et al, 1996), *L. turkestanicus* (Alipour et al, 2004) and with the luciferase sequences of *L. nociluca* (Li et al, 2003a) and *P. rufa* (Li et al, 2003b), it was deduced that there are also six introns and seven exons in the luciferase gene of *D. pectinealis*, which encodes 547 amino acid residues (Fig.1). The intron boundaries are illustrated in Fig. 2.

2.2 Phylogenetic considerations

Compared with the amino acid sequence of known luciferase (Tab. 2), the deduced length of the lu-

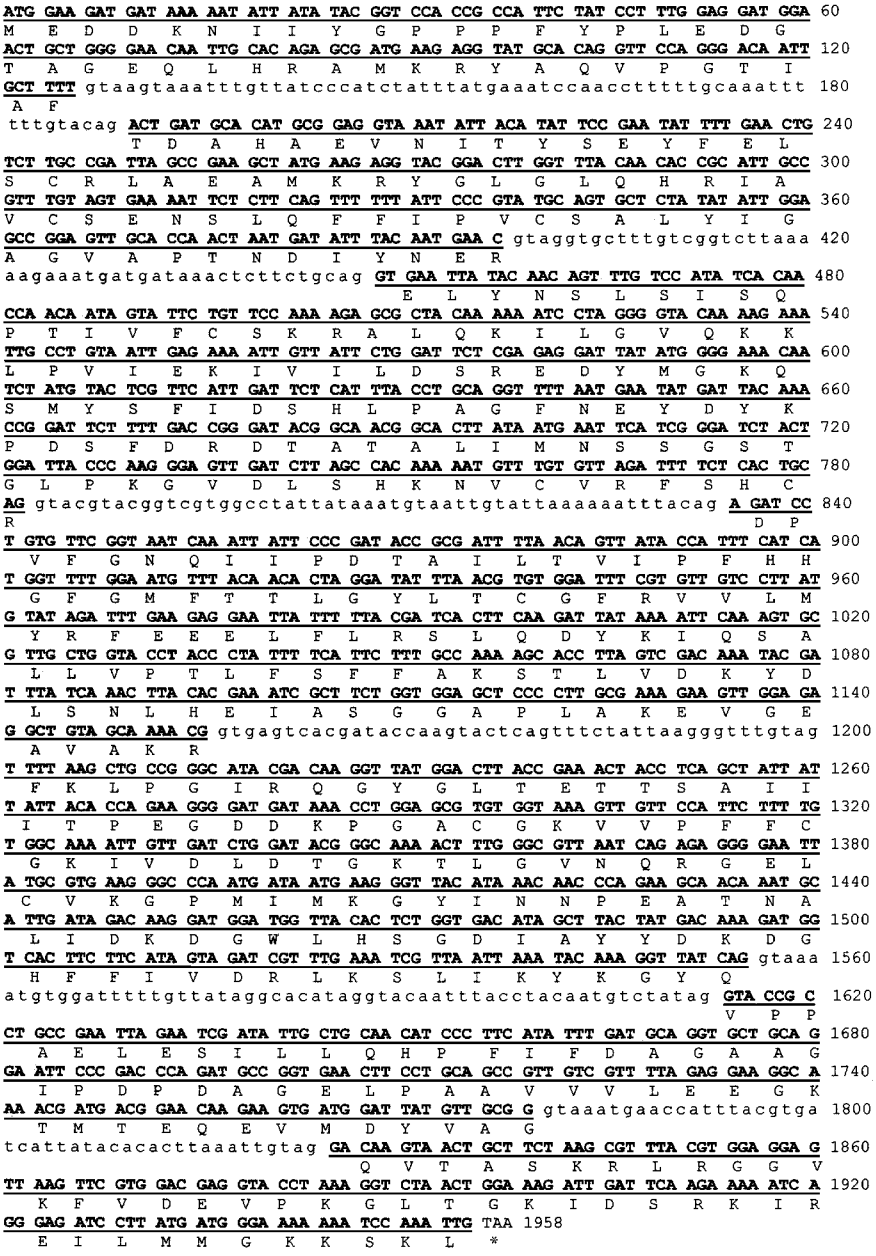
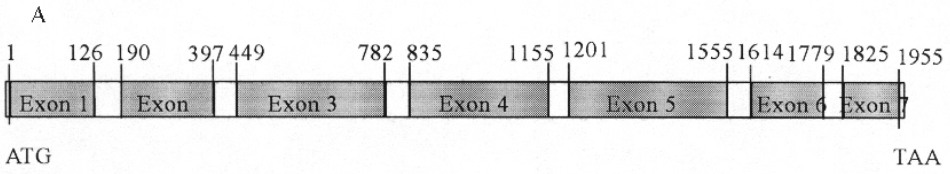


Fig. 1 The nucleotide sequence of the *D. pectinealis* luciferase gene and its deduced amino acid sequence

Nucleotide numbers are presented on the right. Exons are labeled with underlines.

ciferase gene of *D. pectinealis* showed high protein similarity to those of the Lampyrini tribe (93% – 94%) and the Cratomorphini tribe (92%), while the lowest similarity was found with America firefly *P. pyralis* (83%) of the Photinini tribe within the same subfamily Lampyrinae. The protein similarity of luciferase between *D. pectinealis* and Luciolinae species was 67% – 69%. The lowest similarity was found with Photurinae (62%). Furthermore, the phylogenetic analysis of deduced amino acid sequences showed that *D. pectinealis* formed one group with *Lampyris*, *Pyrocoelia* and *Cratomorphus* fireflies with high bootstrap value (BP = 100) both in MP and NJ trees (Fig. 3A and 3B). A small anomaly was that the relationship of *D. pectinealis* and *C. distinctus* was not well supported in the MP tree, but in the NJ tree *D. pectinealis* was well at the base of *Pyrocoelia* + *Lampyris*.

Coding nucleotide sequences only as amino acid characters for phylogenetic analysis may yield misleading results (Simmons & Freudenstein, 2002; Simmons



B

Exon	Length of exon (bp)	Position in gene	Sequence at exon-intron junction
			M E D D ..... I A F
1	126	1-126	ATG GAA GAT GAT ..... ATT GCT TTT gtaagtaaat
			T D A H ..... Y N E R
2	208	190-397	tgtacag ACT GAT GCA CAT ..... TAC AAT GAA C gtaggtgc
			E L Y ..... S H C R
3	334	449-782	cttctgcag GT GAA TTA TAC ..... TCT CAC TGC AG gtacgtac
			D P V ..... V A K R
4	321	835-1155	tttacag A GAT CCT GTG ..... GTA GCA AAA CG gtgagtcac
			F K L ..... K G Y Q
5	355	1201-1555	gggtttgtag T TTT AAG CTG ..... AAA GGT TAT CAG gtaaa
			V P P ..... Y V A G
6	166	1614-1779	gtctatag GTA CCG CCT ..... TAT GTT GCG G gtaaatga
			Q V T ..... S K L
7	131	1825-1955	attgtag GA CAA GTA ACT ..... TCC AAA TTG TAA

Fig. 2　The genomic organization of *D. pectinealis* luciferase gene

A: Exon/intron structures. Numbers mean the length (bp) of exons and introns; B: Lengths of exons and exon /intron boundaries.

Tab. 2　Pairwise identities and similarities of the deduced amino acid sequences among *D. pectinealis* luciferase and other luciferase genes

Animal	Percent similariy																				
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. ***D. pectinealis* (DQ408300)**	–	97	97	98	95	96	95	95	96	90	82	82	81	81	81	81	81	81	81	81	76
2. *L. noticluca* (X89479)	94	–	99	99	97	97	96	96	97	90	82	82	81	81	81	81	81	81	81	81	76
3. *L. noticluca* (Y447204)	94	99	–	99	97	97	96	96	97	90	83	82	81	81	81	81	81	81	81	81	76
4. *L. turkestanicus* (AY742225)	94	98	98	–	97	97	97	97	97	91	82	82	82	81	81	81	81	81	81	81	76
5. *P. rufa* (AF328553)	93	95	95	95	–	99	99	98	95	88	81	80	81	80	80	80	80	80	80	80	75
6. *P. rufa* (AY447202)	93	95	95	95	99	–	99	99	95	89	81	81	81	81	81	81	81	81	81	81	75
7. *P. rufa* (Y447203)	93	95	94	95	99	99	–	98	95	88	80	80	80	80	80	80	80	80	80	80	74
8. *P. miyako* (L39928)	93	95	95	95	98	99	98	–	95	88	80	80	81	80	80	80	80	80	80	80	74
9. *C. distinctus* (AY633557)	92	93	93	93	91	92	91	91	–	90	82	81	81	81	81	81	81	81	81	81	76
10. *P. pyralis* (M15077)	83	84	84	84	82	82	81	81	83	–	82	82	81	81	81	81	81	81	81	81	75
11. *L. lateralis* (X66919)	69	69	69	69	68	68	67	67	69	68	–	98	90	90	90	90	90	90	90	90	74
12. *L. cruciata* (M26194)	68	68	68	68	67	67	67	67	68	68	93	–	90	90	90	90	90	90	90	90	73
13. *L. mingrelica* (S61961)	67	66	66	67	66	67	66	66	66	67	82	80	–	98	98	98	98	98	98	98	73
14. *H. unmunsana* (F486800)	66	66	66	66	66	66	65	65	65	67	81	80	96	–	100	100	100	99	99	99	73
15. *H. unmunsana* (AF420006)	66	66	66	66	66	66	65	66	66	67	81	80	96	99	–	100	100	99	99	99	73
16. *H. papariensis* (AF486802)	66	66	66	66	66	66	65	65	65	67	81	80	96	100	99	–	100	99	99	99	73
17. *H. papariensis* (AF486803)	66	66	66	66	66	66	65	65	65	67	81	80	96	100	99	100	–	99	99	99	73
18. *H. tsushimana* (AF486801)	66	65	65	66	65	66	65	65	65	67	81	80	96	99	99	99	99	–	99	99	73
19. *H. tsushimana* (AF486804)	66	65	65	66	65	66	65	65	65	67	81	80	96	99	99	99	99	99	–	98	73
20. *H. parvula* (L39929)	67	66	66	66	66	66	66	66	66	67	82	81	97	98	97	98	98	98	97	–	73
21. *P. pennsylvanica* (U31240)	62	61	61	61	60	60	60	60	62	59	56	55	53	54	54	54	54	54	54	54	–
	Percent identity																				

et al, 2002; Cameron et al, 2004). Therefore, phylogenetic trees (Fig. 4A and 4B) based on nucleotide sequences of the luciferase gene are also constructed to compare with those of deduced amino acid sequences. Similar to trees of deduced amino acids, the monophyly of Lampyrini + Cratomorphini is recovered in both MP

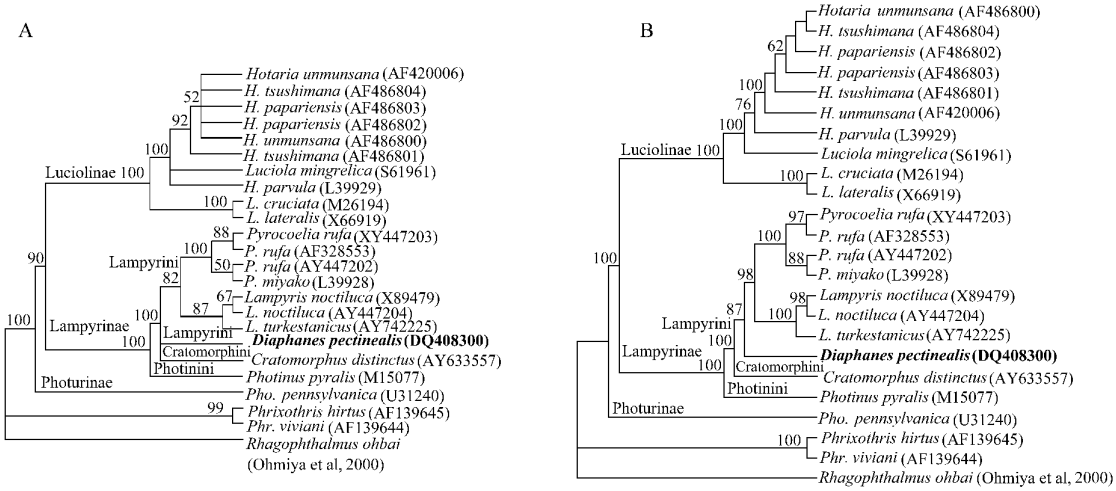


Fig. 3 Phylogenetic trees for aligned amino acid sequences of the *D. pectinealis* luciferase and the known luciferases. A: MP tree; B: NJ tree.

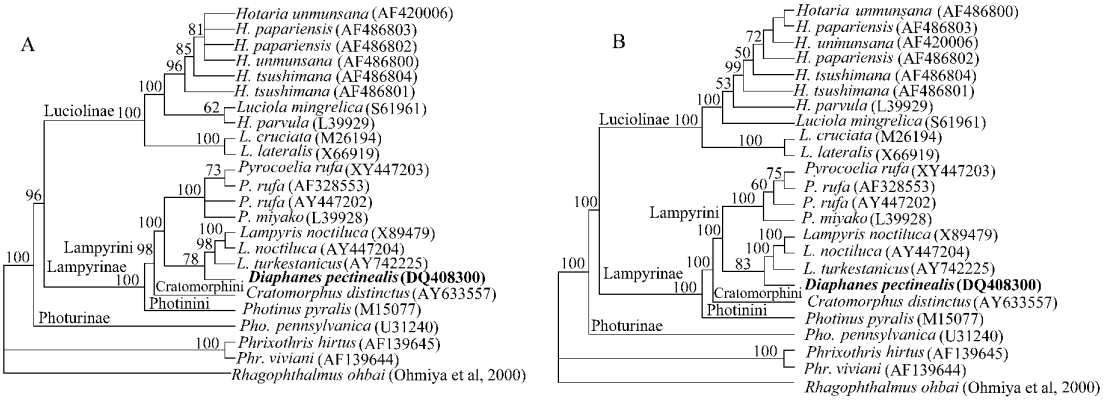


Fig. 4 Phylogenetic trees for aligned nucleotide sequences of the *D. pectinealis* luciferase and the known luciferases. A: MP tree; B: NJ tree.

and NJ trees of nucleotide sequences. However under the level of tribe, the topology from the latter analyses seems more appropriate, in which the tribe Lampyrini forms a monophyly with *D. pectinealis* as a sister to the *Lampyris* group.

3 Disscussion

The phylogenetic analyses based on both the deduced amino acid sequences and nucleotide sequences showed that *D. pectinealis* formed one group with *Lampyris*, *Pyrocoelia* and *Cratomorphus* fireflies with high bootstrap value (BP = 100) both in MP and NJ trees (Fig. 3A and 3B; Fig. 4A and 4B). It was revealed by field observation that *D. pectinealis* emits continuous green bioluminescence. This gives supports to its closeness to the Lampyrini and Cratomorphini fireflies, which also emit continuously green light with emission spectrum peaks usually at 550 nm (Ohmiya et al, 1995; Sala-Newby et al, 1996; Viviani et al, 2004).

The 16*S* mitochondrial DNA analysis also revealed that *D. pectinealis*, together with its congeneric species such as *D. nubilus* and *D. lampyroides*, formed a group with *Pyrocoelia* fireflies (Li et al, 2006). The morphological phylogeny (Branham & Wenzel, 2003) also indicated the close relationship between *Cratomorphus* and *Pyrocoelia*. It is noteworthy that *Diaphanes*, *Lampyris* and *Pyrocoelia*, belonging to Lampyrini and distributed in Asia, Europe and Africa, actually show high morphological similarities to

each other such as elongate clypeus and small mandibles, which are different from those of *Cratomorphus*, placed in Cratomorphini and only limitedly distributed in South America (McDermot, 1964). Nevertheless, it is regretted that the luciferase gene sequences of *D. nubilus* and *D. lampyroides* are still unknown, and no 16*S* mitochondrial DNA sequence of *Lampyris* and *Cratomorphus* fireflies are reported so far, which, to some extent, restricts further explanation of the phylogeny of *D. pectinealis* from the viewpoints of both luciferase and mitochondrial DNA sequence data. Even so, the comparisons of the nucleotide and its deduced amino acid sequences of *D. pectinealis* and other known luciferase genes with reference to morphological, luminous behavioral and mitochondrial DNA data, to some degree enforces the utility of the luciferase gene for phylogenetic analysis at the species or higher taxonomic level.

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第四届海内外华人神经科学研讨会圆满闭幕

“第四届海内外华人神经科学研讨会”于 2006 年 7 月 8 日在云南省丽江市玉龙花园宾馆圆满闭幕。会议期间来自祖国各地(包括香港)、加拿大、英国、美国、韩国、瑞典、德国、日本、澳大利亚、爱尔兰等多个国家和地区的杰出神经科学家们以墙报和口头报告形式,展现了他们在老年痴呆、慢性疼痛、精神疾病、学习记忆、新药研究与开发等领域的最新进展。

此次会议中墙报交流最具特色,会务组给各位代表提供了各种具有云南特色的水果、啤酒、红酒、咖啡、茶水。参会代表们仔细地阅读墙报展览,与墙报作者进行了充分的讨论,气氛热烈。会务组还邀请科学家对墙报进行了评选,选出了 5 名优秀墙报报告人,并在闭幕式大会上给予了奖励。

会议的另一个特色是口头报告人的幻灯片制作非常精美、图文并茂、形象生动。除了外籍神经科学家们用英语做学术报告外,海外华人神经科学家们几乎都用汉语成功、精彩地做了学术报告。值得强调的是,许多海外华人神经科学家们长期使用英语,对汉语尤其是专业术语并不熟悉,但是他(她)们努力地克服了这种困难,报告前利用各种机会学习和确认自己将报告内容中的中文名词。因此幻灯片制作虽然采用了英语,但会议报告几乎都用了汉语;且绝大多数华人代表能用母语进行热烈地讨论和交流,有的代表更是幽默风趣,使一些会场欢笑声不断,气氛异常活跃。

脑健康和脑疾病成为了参会代表们的热门话题,帕金森、老年痴呆症,抑郁症等疾病的细胞模型和动物模型等都备受大家关注。在这些模型的基础上疾病机制和治疗药物的研发成为了大会讨论的焦点。由于会议报告时间安排紧凑,15 分钟的报告时间和 5 分钟的讨论时间都无法满足参会代表们的需求,会议会场主席经常都提醒大家“提问和回答均要精练”。

此次会议还增加了讨论会场。代表们主要就国内外基金申请、新药研发和我国神经科学发展展开了讨论。代表们争先恐后发言,各抒己见。来自美国 NIH 的两位基金项目官员向大家介绍了在美国的基金申请流程和现状,并对比了我国基金申请的情况。鼓励海内外的华人神经科学家们合作,联合一起申请国际资金项目。在新药研发的讨论中,许多专家指出了欧美和韩日等国已率先将我国的一些中医药瑰宝开发成为了新药并申请了专利;敦促到会的华人神经科学家们要利用我们自己的优势开发新药,保护我国的中医药遗产。参会代表为我国的神经科学事业的发展踊跃地献计献策。最后会议通过无记名投票选举了下一届——第五届海内外华人神经科学会的会议组委会成员:王玉田(加拿大)、钟毅(美国)、徐志卿(瑞典)、何士刚(中科院生物物理所)、唐北沙(中南大学湘雅医院)。

此次会议的开幕式是在云南省昆明市世博花园宾馆举行的。中国科学院昆明动物所所长张亚平院士、云南省科技厅王建华副厅长先后致欢迎词。开幕式简短、浓重热情。而丽江会场闭幕式则是活跃非凡。海内外许多代表包括加拿大科学院院士登台高歌,以自编的歌词感谢会议会务的组织安排、感谢丽江美丽多彩的自然风光和民族文化。闭幕式整整持续了 2 个多小时,代表们既观赏了丽江少数民族歌舞,又品尝了美味佳肴,还领略了华人神经科学家们的多才多艺和丽江人民的热情奔放。第四届海内外华人神经科学会在掌声和欢笑声中、在代表们轻唱着“友谊天长地久”中闭幕。

(备注:会议论文集已由高教出版社出版为电子图书《神经科学进展四》,在全国发行。)

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